Determination of Triglyceride Composition of Fats

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THE PHYSICAL PROPERTIES and performances of fats are directly related to their glyceride composition and structure. Hence knowledge of these compositional factors is important in connection with research aimed at improvement of fat-products for specific uses. Sufficient qualitative evidence was obtained by early investigators prior to 1900 to conclude that fats and oils in general were composed of mixtures of mixed glycerides rather than mixtures of simple glycerides. The first comprehensive investigation in which chemical methods and techniques were applied in attempts to obtain quantitative information on glyceride composition of fats and oils was initiated about 1927 by Hilditch and his collaborators (13, 4, 5). Their studies have continued up to the present and have included numreous fats and oils of vegetable and animal origin. An extensive review of work in this field up to 1947, much of which has been done by Hilditch and coworkers, has been published (10).

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The scope of this paper is restricted to a discussion of methods and techniques which are being used to determine glyceride composition. An attempt was made to outline several concepts on glyceride distribution in natural fats in relation to experimental data.

Tri-saturated glycerides. Probably the first method for determining tri-saturated glycerides in fats is that of Hilditch and Lea (13). They showed that glycerides containing unsaturated acid radicals are oxidized to azelao-glycerides when treated in acetone solution with excess of powdered potassium permanganate. The saturated acid radicals are not affected. Thus the oxidation of a fat containing GS_3,[2] GS_2U, GSU_2, and GU_3 would result in a product containing unchanged GS_3, and GS_2A,[2] GSA_2, and GA_3. By careful treatment of the oxidation product with aqueous alkali the acidic azelao-glycerides can be extracted from the GS_3, the latter determined simply by weight. If any unsaturation remains with the GS_3 portion, the fraction is again subjected to the oxidation and extraction. The method is tedious and time-consuming, and troublesome emulsions are often encountered.

Another method, based on crystallization of fats from acetone and application of appropriate calculations, has also been employed to advantage for determining GS_3 (32, 33, 14). In this method the fraction containing the GS_3 also contains significant amounts of the most insoluble members of the GS_2U. Correction for the amount of the latter can be made after the fatty acid composition has been determined. This method however would probably give low values for GS_3 if the fat contained large amounts of saturated acids of lower molecular weight than that of palmitic acid, owing to increased solubility.

Cama *et al.* (3) listed the GS_3 contents determined on a number of fats by both methods. The saturated acid content of each fat was also shown. This information is reproduced in part in Table I. In general, the agreement is exceptionally good, particularly considering that the values were obtained by different workers in most instances and on different specimens of fat.

TABLE I

Trisaturated Glycerides of Fats Determined by Oxidation and Crystallization Methods (3)

Fats	Oxidation Method		Crystallization Method	
	Sm[a]	GS_3	Sm[a]	GS_3
		% mol		% mol
Coconut	93	84	94	82
Palm kernel	85	66	87	62
Stillingia tallow	68	24	73	21
Borneo tallow	63	5	63	5
Cacao butter	60	3	61	2
Kokum butter	59	2	59	1
Palm oil, Cameroons	49	8	53	8
Palm oil, Belgian Congo	50	6	50	6
Sheep	61	27	61	28
Cow, English	58	18	59	17
Pig, perinephric	51	11	51	9
Pig, back	43	7	44	5
Buffalo (Indian) milk fat	75	42	72	40
Cow (Indian) milk fat	68	34	69	35

[a] Sm = % (mol) of saturated fatty acids.

Determination of principal types of glycerides. The number of possible combinations of the fatty acids with glycerol to form triglycerides increases geometrically with each additional fatty acid, *i.e.*, it equals n^3 where n is the number of different fatty acids (31). The number of chemically distinguishable glycerides is ½ (n^3 plus n^2).

Most natural fats contain four or more component acids and therefore may have 40 or more chemically distinguishable glyceride entities. These individual glycerides may differ by only a few methylene groups or in degree of unsaturation, and have molecular weights usually greater than 800. Hence it is not surprising that the complete elucidation of glyceride composition of fats remains an unsolved problem. Some progress however is being made. Several procedures have been developed which permit an estimation of the proportions of the four principal types, i.e., GS_3, GS_2U, GSU_2, and GU_3, and in some of the simpler fats an approximation of the major individual component glycerides.

Crystallization methods. The procedures employed by Hilditch and collaborators, particularly in their later glyceride composition studies, will be outlined briefly. The fat or oil is separated first into a number of fractions by crystallization from acetone. Depending on the nature and complexity of the fat, the number of final fractions may vary about 3 to 6 or more and the temperatures of crystallization from about 20° to −60°C. One of their more elaborate systematic crystallization procedures has been described by Hilditch and Maddison (14) for investigation of glyceride components of cottonseed oil.

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[2] Throughout this paper GS_3, GS_2U, GSU_2, and GU_3 will be used to represent tri-, di-, and monosaturated, and triunsaturated glycerides, respectively. Similarly GS_2A, GSA_2, and GA_3 will represent corresponding azelao-glycerides.

The original fat and each fraction obtained from it are analyzed for individual fatty acid content, iodine value, and saponification equivalent. In some cases where the fraction is small, fatty acid composition may be computed from iodine value, thiocyanogen value, and saponification equivalent. In large fractions the ester fractionation method with appropriate analyses of each distilled fraction is employed. Again, depending on the fat or oil, certain fractions may be saponified and separated into "solid" and "liquid" acids by the lead salt alcohol method, followed by appropriate analyses or possibly by fractional distillation of the methyl esters of the solid and liquid acids and analyses of the fractions. From the fatty acid analysis of each fraction obtained in the crystallization of the fat, molar increments of each fatty acid are computed. From these data the molar increments of each of the four general types of glycerides can be estimated on the assumption that no more than two types are present in any one fraction. The summation of the increments in each fraction of each type of glyceride is the estimated percentage occurring in the fat.

[In some instances where a less elaborate crystallization system was employed, portions of the fractions were also completely hydrogenated, and the hydrogenated sample was crystallized to fractionate tri-C_{18} (as tristearin). Calculation from saponification equivalents on the crystallized fractions gave an estimated tri-C_{18} content, which together with the fatty acid analysis before hydrogenation, permitted more reliable estimates to be made concerning the total tri-C_{18} unsaturated glycerides.]

Luddy *et al.* (33) described a systematic crystallization procedure which was somewhat simpler and employed considerably different solvent ratios and temperatures. They determined the unsaturated fatty acid components by means of improved spectrophotometric methods and iodine values. The saturated were obtained by difference. Results on a number of fats reported by them are given in Table II along with values reported by Hilditch and collaborators. A strict comparison of the two crystallization procedures cannot be made because different samples of the same type of fat were used by the several workers.

TABLE II

Glyceride Composition as Determined by Different Crystallization Procedures on Different Fats

	Iodine Value	Saturated Acid	Principal Glyceride Types			
			GS_3	GS_2U	GSU_2	GU_3
		% mol	% mol	% mol	% mol	% mol
Cottonseed oil (14)	105.0	28	0.1	13	58	28
Cottonseed oil (33)	115.2	27	none	14	51	34
Palm oil (15)	53.0	53	8	54	32	6
Palm oil (33)	50.1	55	9	48	39	3
Pig back fat (19)	59.2	44	5	32	60	3
Lard (33)	66.2	39	3	27	55	15
Chicken fat (20)	79.5	34	2	28	41	29
Chicken fat (33)	78.5	30	2	18	49	31

Cama *et al.* (3) made a reasonable attempt to evaluate the crystallization procedure by making several artificial mixtures of the four principal types of glycerides. Fractions, which had been obtained by intensive application of the crystallization technique to different fats, were analyzed for fatty acid composition and calculations were made of their glyceride composition. None of the fractions were composed of one type only but invariably contained two neighboring types, *e.g.*, GS_3+GS_2U, GS_2U+GSU_2, GSU_2+GU_3. From these fractions of calculated compositions three artificial fat mixtures of widely varying composition were prepared and subjected to the systematic crystallization technique, followed by fatty acid analysis of the crystallized fractions. The "found" values are compared with the calculated ones in Table III along with "found" and calculated values for a 50/50 mixture of poppy-seed oil and sesame oil whose glyceride compositions had previously been determined by the crystallization procedure.

TABLE III

Glyceride Composition of Artificial Mixtures of Fat Components Determined by Crystallization Technique (3)

	Fat Mixtures							
	A		B		C		Poppy/Sesame 50:50	
	Found	*Calc.*	*Found*	*Calc.*	*Found*	*Calc.*	*Found*	*Calc.*
GS_3	17	17	5	5	....	....	....	....
GS_2U	47	47	41	34	1	5	3	3
GSU_2	36	36	52	59	50	42	41	43
GU_3	....	....	1	2	49	53	56	54

Although the accordance was excellent in mixture A, and good in the poppy-sesame mixture, the GS_2U and GSU_2 contents found for B did not accord well with the calculated values. The distribution in C was also not in good agreement with the calculated. Cama *et al.* however do not feel that the artificial mixture analyses represent an altogether satisfactory test of the method because the preparation and storage of the fractions from which the mixtures were made involved risk of oxidative changes over and above that normally incurred in the crystallization method as applied to a fresh natural fat or oil.

Oxidation method. Kartha (25, 26, 27) modified the oxidation method for GS_3 *(loc. cit.)* and employed it for the determination of the four principal types of glycerides. He found that if a slight excess of acetic acid is maintained during the oxidation with permanganate, hydrolysis of the azelao-glycerides is prevented. He further found that by treatment of the oxidation product with magnesium sulfate solution, GS_3, all of the GS_2A and part of the GSA_2 (as magnesium salts) could be obtained as a precipitate while the balance of the GSA_2 and GA_3 (as magnesium salts) was contained in the filtrate. After treatment with mineral acid the recovered product from the precipitate was weighed and saponified; saturated acids were determined by the Bertram technique. Based on the weight of the precipitated fraction before saponification, the amount of GS_3 (determined independently) and the weight and neutralization number of the saturated acids, calculations can be made of the total GS_2A and the amount of GSA_2 in this fraction. The balance of the GSA_2 is determined from the saturated acids isolated in similar manner from the soluble fraction of magnesium salts. The total GS_2A and GSA_2 can then be calculated to equivalent amounts of GS_2U and GSU_2 and expressed in percentage (mol) of the whole fat. The GU_3 is obtained by difference.

The principal types of glycerides have been determined by both experimental methods on a number of dissimilar fats and oils. Only in a few instances however have both methods been applied to the same specimen of fat as shown in the footnote for Table IV. The accordance between the two methods in these instances was reasonably good except for palm oil (33). In another analysis of palm oil (15) the agreement was satisfactory. How much of the lack of accordance in the results on the other fats shown in the table is attributable to variation between different specimens of the same type of fat, of course, is un-

TABLE IV

Glyceride Composition of Fats and Oils Determined by Crystallization and Oxidation Methods

	Lit. Ref.	Sm[a]	Crystallization Method				Lit. Ref.	Sm[a]	Oxidation Method			
			GS_3	GS_2U	GSU_2	GU_3			GS_3[b]	GS_2U	GSU_2	GU_3
Groundnut (peanut) oil	(9)	19	....	1	56	43	(27)	20	....	9	42	49
Cottonseed oil	(14)	28	....	13	59	28	(27)	23	....	13	44	43
Cottonseed oil[c]	(33)	27	....	14	51	35	(33)	25	....	13	48	39
Neem oil	(16)	32	....	14	67	19	(27)	40	....	39	41	20
Mowrha oil	(12)	43	1	28	71	0	(27)	43	trace	47	36	17
Palm oil[c]	(33)	55	9	48	39	3	(33)	51	9	47	31	13
Palm oil	(15)	53	8	54	32	6	(27)	54	9	54	27	9
Vateria Indica fat	(40)	58	2	69	29	0	(27)	57	1	76	18	5
Garcinia Indica fat	(17)	59	1	76	21	2	(27)	61	1	84	13	2
Ox depot fat	(18)	61	17	49	34	0	(27)	63	17	58	22	3
Lard[c]	(33)	39	3	27	55	15	(33)	39	3	25	59	14
Chicken fat[c]	(33)	30	2	18	49	31	(33)	29	2	18	44	35

[a] Saturated fatty acids % (mol).
[b] Determined by independent method.
[c] Both methods applied to same specimen of fat.

known. It appears unlikely however that two specimens of the same type of fat or oil having the same percentage of saturated acids, such as Mowrha oil, would vary greatly in glyceride distribution. Therefore, perhaps for the most part, the discrepancies should be attributed to inadequacies of the methods.

There are a number of sources of error in either the crystallization or oxidation method. In the former the calculation of the principal types is based on the assumption that only two neighboring types are present in any one fraction. There may be more overlapping in solubility between certain members of the GS_2U and GSU_2 than is realized. Hence the filtrate fractions (obtained from the lower-temperature crystallizations), which are normally calculated as a mixture of GU_3 and GSU_2, may contain significant amounts of the most soluble members of GS_2U. The amount of the latter calculated as GSU_2 would be doubled and would produce a correspondingly lower GU_3 content (by difference). The reliability of the crystallization method also depends on the accuracy of the fatty acid determinations of each fraction. In the calculation of glyceride types for a given fraction, only the percentage of total saturated or (total unsaturated) fatty acids is used. Errors in fatty acid determination in some instances may be trebled in computing to glyceride basis.

There are also a number of possible sources of error in the oxidation method. Assuming strictly quantitative techniques, the accuracy of the method depends also on the following: a) that no unsaturated acids are present with a double bond closer to the carboxyl than the 9,10-position; b) that no hydrolysis takes place during the oxidation or subsequent treatment of the oxidation product including the extraction, washing, magnesium soap precipitation, decomposition etc.; and c) that the GS_2A is completely precipitated as magnesium salts and that the precipitate contains no GA_3 (as magnesium salts). Furthermore the saturated acids are determined by the Bertram technique after saponification of the azelao-glycerides recovered from the magnesium salt precipitation. This technique is known to give low results when acids lower in molecular weight than palmitic acid are present.

The recent developments of apparatus for multiple stage countercurrent extraction have furnished a new tool which offers considerable promise in the fractionation and resolution of glyceride mixtures. Dutton (6) has recently reviewed the principles and applications of countercurrent extraction for the fractionation of lipids. Its application is still in a preliminary status, but sufficient data have been reported to demonstrate potentialities for the analytical separation of glycerides of fats and oils.

Concepts of Glyceride Distribution in Natural Fats

The mode of synthesis and elaboration of fats by plants and animals has been a subject of great interest for many years. As more definite information became available concerning the glyceride composition of a wide selection of fats from different sources, it was inevitable that attempts would be made to determine whether the biosynthesis followed any distinct pattern.

Hilditch and collaborators were first to suggest a pattern of distribution. The suggestion originated as a broad generalization to account for certain observations, such as the following.

1. With few exceptions natural fats and oils contain a minimum rather than a maximum of simple triglycerides, *i.e.*, where all three fatty acids of the glyceride are the same.
2. In a number of liquid seed fats, in which oleic and other unsaturated acids predominate, trisaturated glycerides are essentially absent while the triunsaturated content was uniformly closer to the least possible (based on calculations from fatty acid compositions), thus indicating a maximum association of saturated and unsaturated acids in the form GS_2U and GSU_2.
3. Seed fats contain only minor proportions (a few %) of GS_3 unless the percentage of saturated acids exceeds about 58%.
4. In seed fats containing over 58% of saturated acids, the "association ratio" (mols saturated acids/mols unsaturated acids) of the saturated-unsaturated glyceride portion is nearly constant at about 1.2-1.4 to 1. This ratio corresponds with a mixture of about 3 to 4 (mols) of GS_2U to 1 (mol) of GSU_2.

To account for these observations they proposed that the component acids of a fat tend to be distributed, according to their relative proportions, as evenly or widely as possible among all the glyceride molecules. The operation of this principle in its strictest sense would lead toward maximum heterogeneity in any individual glyceride but not in the fat as a whole.

As more data on the glyceride composition of a wider selection of fats became available, the proposal was modified to bring it in better conformity with the observed compositions and was expressed in the following four general terms, which have become known as the "rule of even distribution" (11): a) when a given fatty acid A forms about 35% (mol) or more of the total fatty acids (A+X) in a fat, it will occur at least once, $G(AX_2)$ in practically all the triglyceride molecules of the fat in question; b) if it forms from about 35 to about 65% (mol) of the total fatty acids, it will occur twice $G(A_2X)$ in any given triglyceride molecule in some instances, and, of course, more frequently the higher the proportion of this acid in the total fatty acids; c) if it forms 70% or more of the total fatty acids, the remaining fatty acids (X) can at most only form mixed triglycerides $G(A_2X)$, and the excess of A then, and

broadly speaking then only, appears as a simple triglyceride, $G(A_3)$; and d) a minor component acid which forms much less than about a third of the total fatty acids (*e.g.*, 15% or less) will not occur more than once in any triglyceride molecule (and, of course, not at all in many).

An empirical system of computing the approximate proportions of the major glyceride components in fats composed of three (or at most four) major component fatty acids was also introduced. It has been applied principally to fractions derived from crystallizations of fats and consists of arithmetical proportioning of a major component acid (usually oleic acid or oleic + other unsaturated acids) to the other major component acids on the basis of their molar proportions, and then each portion of "oleic" acid is calculated as combinations with the other major components. An example of the calculation as applied to cacao butter is cited (10). The molar proportions of acids in the whole fat were palmitic, 24.3; stearic, 35.4; oleic, 38.2; linoleic, 2.1. The oleic and linoleic are combined for the purpose of calculation as 40.3% (mol). This amount of "oleic" acid is divided with palmitic and stearic acids: $\frac{24.3}{24.3+35.4}\times 40.3=16.4\%$ (mol) "oleic" to combine with 24.3% (mol) of palmitic acid, the balance of "oleic," 23.9% (mol), to combine with 35.4% (mol) of stearic acid.

These combinations as mono- and di-"oleins" are calculated as follows:

$x+y=16.4+24.3=40.7$

$1/3x+2/3y=16.4$

$y=8.5\%$ (mol) di"oleo"monopalmitin (OOP)

$x=32.2\%$ (mol) mono-"oleo"dipalmitin (OOP)

Similarly, combinations of the "oleic" and stearic acids give 12.4% (mol) di-"oleo"-stearin (OOS), and 46.9% (mol) mono-"oleo"-distearin (OSS). Experimental and calculated values are shown in Table V.

The assumptions made in this type of calculation, of course, would have considerable influence on the results, as shown by the comparison of calculated with found values for cottonseed oil (10) in Table VI. The molar proportions of fatty acids in the oil were: myristic, 2.4; palmitic, 24.4; stearic, 1.6; oleic (with lower homologs), 24.9; and linoleic, 46.7. The distribution of the four principal types of glycerides was determinel by the crystallization method as outlined previously. The calculations of major "individual" components a), b), c), d) were made from fatty acid analyses of each fraction but with different assumptions. Calculations e) and f) were made from fatty acid composition of the original oil. The composition of the oil is considered most likely to be represented by a), with b) as a possible second choice.

It is apparent however that calculations made directly from fatty acid composition of the original oil are hardly a good approximation of the found values or of the values calculated from fatty acid composition of the crystallization fractions.

TABLE V

Component Glycerides of Cacao Butter

	Experimental	Calculated
	% mol	% mol
Dipalmitostearin	2	....
"Oleo"-dipalmitin	6	....
"Oleo"-palmitostearin	52	64.4
"Oleo"-distearin	19	14.7
Di-"oleo"-palmitin	9	8.5
Di-"oleo"-stearin	12	12.4

TABLE VI

Comparison of Observed Glyceride Composition of Cottonseed Oil With Values Calculated on Different Assumptions

Component Glycerides		Found	Calculated [% (mol)]					
			(a)[a]	(b)[a]	(c)[a]	(d)[a]	(e)[b]	(f)[b]
		% mol						
GS_3		0.1						
	PPP	0.1	0.1	0.1	0.1	0.1	....	....
GS_2U		13.2						
	OSS		5.9	5.9	5.9	3.8	....	2
	LSS		7.3	7.3	7.3	9.4	4	5
GSU_2		58.4						
	OOS		....	....	15.5	21.4	....	....
	SOL		40.6	50.7	35.2	....	56	53
	SLL		17.8	7.7	7.7	37.0	21	22
GU_3		28.3						
	OOO		....	....	0.9	....	....	....
	OLL		28.3	18.2	....	28.3	19	18
	LLL		....	10.1	27.4	....	....	....

[a] Calculations based on fatty acid analyses of fractions from systematic crystallizations (14).
(a) maximum possible OLL, then SOL
(b) maximum possible SOL
(c) maximum possible SOL, then LLL
(d) maximum possible SLL, then OLL

[b] Calculations made from fatty acid composition of original oil.
(e) linoleic acid proportioned between palmitic, stearic (+ myristic), and oleic acids.
(f) oleic acid proportioned between stearic, palmitic, and linoleic acids.

Fruit coat fats, in general, show greater divergence from the terms of even distribution than the seed fats. They have greater contents of GS_3 than would be expected under the terms. In some instances, at least, further arbitrary assumptions had to be introduced into the system of calculation in order to proximate the glyceride composition found experimentally. For example (15) in palm oils (Cameroons and Bassa), the GS_3 and GU_3 were calculated on the "random" basis, and only half these amounts were taken as the "computed" values. Further assumptions were made concerning the composition of the "half-random" values for GS_3 and GU_3. After subtracting the molar percentages of the acids representing the "computed" GS_3 and GU_3 from the total amount of these acids in the oil, the GS_2U and GSU_2 contents were estimated by partitioning the remaining oleic acid among the remaining palmitic, stearic, and linoleic acids in the usual way.

Land animal depot fats, particularly those containing appreciable amounts of stearic acid, according to Hilditch, represent a special case which deviate from even distribution only in that greater amounts of GS_3 are present in relation to the proportion of saturated acids in the fat. When the saturated acids represented in GS_3 are deducted from the total of these acids present in the original, the system of calculation based on partitioning "oleic" acid between the rest of the palmitic and stearic acids gave values for GS_2U, GSU_2, and GU_3 which were in accord with those found experimentally by Hilditch and Pedelty (19).

Random distribution. It has been observed that the distribution of the principal types of glycerides in many fats conforms as closely to values calculated on the basis of random distribution as to those calculated on the terms of even distribution, particularly where the latter calculation is applied in its strictest sense to the fatty acid composition of the original fat.

The calculation of the principal glyceride types on the basis of random or indiscriminate distribution of fatty acids on the glyceride molecules has been discussed previously (42). The assumption is made that in the biosynthesis of fats (or subsequent biochemical

TABLE VII

Comparison of Observed (Found) Glyceride Composition With Values Calculated by Several Hypotheses

Fat	Lit. Ref.	Sm.	GS_3	GS_2U	GSU_2	Gu_3
		% (mol)	% (mol)	% (mol)	% (mol)	% (mol)
Peanut Oil (found)	(25)	20	0	9	42	49
Even	Calc.[a]	20	0	15	31	55
Random		20	1	10	38	51
Restricted Random	(25)	20	0	10	40	50
Cottonseed Oil (found)[b]	(33)	26	0	14	49	37
Even	Calc.[c]	26	0	14	52	34
Random		26	2	15	43	40
Restricted Random		26	0	18	43	39
Cacao butter (found)	(10)	60	2	77	21	0
Even	(10)	60	....	78	22	0
Random		60	22	43	29	6
Restricted Random		60	(2)	78	19	1
Vat. Indica fat (found)[d]	(40, 25)	57–58	2	72	24	2
Even	Calc.[e]	57	0	71	29	0
Random		57	18	42	32	8
Restricted Random		57	(2)	70	25	3
G. Indica fat (found)[d]	(18, 25)	59–61	1	80	17	2
Even		60	0	83	17	0
Random		60	22	43	29	6
Restricted Random		60	(2)	78	19	1
Palm Oil (found)[d]	(15, 25, 33)	53–54	9	52	33	6
Even	Calc.[f]	53	(9)	57	18	16
Random		53	15	40	35	10
Restricted Random		53	(9)	50	33	8
Lard (found)	(33)	39	3	26	57	14
Even	Calc.[f]	39	(3)	37	35	25
Random		39	6	28	43	23
Restricted Random		39	(3)	33	43	22
Tallow (found)	(34)	60	18	47	30	5
Even	Calc.[g]	60	(18)	44	38	0
Random		60	21	43	29	7
Restricted Random		60	(18)	48	28	6

[a] Oleic acid distributed between saturated and linoleic acids.
[b] Average values on same specimen by crystallization and oxidation methods.
[c] Linoleic acid distributed between oleic and saturated acids.
[d] Average values on different specimens by crystallization and oxidation methods.
[e] Oleic acid distributed between saturated acids.
[f] Oleic acid distributed between saturated acids and linoleic acids after subtracting saturated in GS_3 (found) from total saturated acids.
[g] Oleic acid distributed between saturated acids after subtracting the saturated acids in GS_3 (found) from total saturated acids.

processes, such as enzymatic ester interchange) each step is governed by laws of chance. The distribution of the four types of glycerides then is conveniently expressed as the terms in the binomial expansion of $(S+U)^3=1$, or $S^3+3S^2U+3SU^2+U^3=1$, where S = saturated acid and U = unsaturated acid — in mol fractions.

The suggestion of random distribution of glycerides in fats has been confined principally to animal depot fats (38, 7, 31) although it has also been proposed for babassu fat, a palm seed fat (24).

A comparison of the distribution of principal glyceride types found experimentally with those calculated by several hypotheses are shown in Table VII.

Restricted random distribution. An interesting new hypothesis concerning the distribution of glycerides in fats has been proposed by Kartha (25, 27, 28). It is based "on the assumption that there is a maximum proportion of GS_3 which may be present in each species of fat. This limit varies, according to circumstances, up to the proportion of GS_3, which may be produced by random or chance distribution of the component fatty acids among the glyceryl radicals. When the proportion of GS_3 which can exist is less than that which could be synthesized by chance distribution of the saturated fatty acids, the excess S is distributed according to chance among the remaining glyceryl radicals without formation of any more GS_3."

The chance (or random) values for the four principal glyceride types can be calculated as previously indicated, providing the molar proportions of saturated acids in the total fatty acids are known or determined.

The restricted random calculation can then be made from the calculated random values, providing the GS_3 content of the fat is known or determined, as follows:

$GS_3 = GS_3$ actual (Kartha prefers a method to be described (29)

$GS_2U = GS_2U$ chance $+ (GS_3$ chance $- GS_3$ actual) $+ 3a$

$GSU_2 = GSU_2$ chance $- 3a + 3b$

$GU_3 = GU_3$ chance $- 3b$

where a = % of S substituting for U in GSU_2

and b = % of S substituting for U in GU_3

Calculation for a:

$$a = \frac{1/3(GS_3 \text{ chance} - GS_3 \text{ actual}) \times 2/3\ GSU_2 \text{ chance}}{2/3\ GSU_2 \text{ chance} + GU_3 \text{ chance}}$$

Calculation for b:

$$b = \frac{1/3\ (GS_3 \text{ chance} - GS_3 \text{ actual}) \times GU_3 \text{ chance}}{2/3\ GSU_2 \text{ chance} + GU_3 \text{ chance}}$$

A comparison of glyceride distribution determined experimentally with calculated distribution, assuming several hypotheses, are shown in Table VII. Values calculated on the restricted random principle show best accordance with found values throughout this series. Kartha (28) has published a comparison of found values with values calculated on the restricted random principle for a greater number of fats in which good agreement was shown.

Discussion. Inasmuch as the experimental methods for determining glyceride structures leaves much to be desired as to precision, it would be presumptuous to consider that the case is proven for any one distribution hypothesis. Perhaps more important is the fact that even within the limitations of the methods a great deal of knowledge concerning the glyceride structures of natural fats has been accumulated. With more efficient fractionation which may be obtainable with multiple-stage, countercurrent extraction or possibly with new chromatographic techniques, it seems probable that the answer to the question of natural pattern of distribution may soon be forthcoming.

The experimental values reported in Table VII for the most part are average values for a given type of fat reported either by different investigators or by the same investigator who employed both oxidation and crystallization methods. Where values on different specimens of a fat were averaged, the fat contained

approximately the same fatty acid composition, and averages of these were also used in the calculations. Even allowing for the limitations of the experimental data, the values calculated by the restricted random principle are in best accordance for the entire series of fats shown. The method of computing distribution, in either "even distribution" or "restricted random" hypothesis, is designed to bring the values in closer agreement with the observed. Hence it is not surprising that in some instances either hypothesis appears valid.

There are other points to be considered in the question of pattern of distribution aside from the proportions of the four principal classes of glycerides. In oils, such as cottonseed oil, which have a high proportion of linoleic acid (about 50% in many specimens), there should be about 12.5% of trilinolein present according to random distribution. Only evidence for minor proportions has been reported.

Hilditch and Stainsby (21) in fractional crystallization of hydrogenated pig back fat found β-palmitodistearin (M.P. 67–67.5°) in amounts corresponding to about 80% of the total palmitodistearin present in the fully saturated portions and concluded that the original fat contained β-palmityl glycerides unaccompanied by any appreciable quantities of α-palmityl glycerides. This was later confirmed by Meara (37), and more recently by Quimby *et al.* (39). The latter provided evidence, based on cooling curves and X-ray diffraction studies of hydrogenated fractions obtained in crystallization and of hydrogenated whole fats, which indicated that lard contains principally 2-palmityl glycerides while beef and mutton tallows contain largely 1-palmityl glycerides. They pointed out that this evidence does not support the hypothesis of random distribution because the 2-palmityl configuration is only half as probable as a 1-palmityl configuration.

Further evidence of the lack of randomness in lard was furnished by Luddy *et al.* (34), who showed that after treatment with sodium methylate there was a distinct change in glyceride distribution and in physical properties. The glyceride composition after the treatment was in closer agreement with values calculated for random distribution.

Reiser and Diekert (41) made use of an isotope dilution technique for determining GS_3 and found that endogenous rat fat conformed to the random type distribution but the ingested fat appeared to be "resynthesized by the rat according to "even" type distribution, or at least, in a manner which tends to distribute the fatty acids." From similar studies on chicks they found the percentage of GS_3 higher than expected for random distribution. These authors also cited work (1, 35, 30, 41, 8) which showed selective or specific activity of enzymes in the synthesis and hydrolysis of glycerides, a point said to be in favor of non-randomness. However they also state that it is possible for tissues to have many fat-splitting and fat-synthesizing enzymes with different reaction rates and specificities, a condition which would tend toward randomization.

Kartha (28) has offered a plausible explanation to account for the GS_3 content (actual) usually being less than that calculated on a true random basis. He proposed that GS_3 can be synthesized in the depots only to the extent that it can remain in a fluid state. The amount of GS_3 that will remain in a fluid state would vary with the melting point and solubility of the GS_3. The melting point and solubility, of course. would vary with the proportions of low and high molecular weight saturated acids present.

Probably solution of the GS_3 in the liquid saturated-unsaturated glycerides present in the depot fat governs the fluidity rather than melting in the usual sense. Other factors, such as the solubilizing and emulsifying effect of phospholipids, lipoproteins, etc., probably also influence the "fluidity." Hence, in related fats, *e.g.,* in land animals, even though there may be wide differences in fatty acid and glyceride composition between species (and also between individuals of the same species probably due to differences in diet), the depot fats with large GS_3 content should contain greater amounts of the more soluble, lower molecular weight acids. Kartha *(loc. cit.)* has listed a number of fats with pertinent data on their saturated acid composition, GS_3 actual and calculated, and melting ranges, which fall in line with this hypothesis.

The specificity of enzymes and relative rates of enzymatic esterification of different fatty acids with glycerol and rates of hydrolysis of different fatty acid esters of glycerol are important in consideration of glyceride distribution in natural fats. In this connection published information is not conclusive. Artom *et al.* (2) found no difference in the rate of hydrolysis due to α- or β-position of a fatty acid in studies on β-palmityl α,α′-dicaprylin and β-caprylyl α,α′-dipalmitin. Similarly Weber *et al.* (43) found the α- and β-monoglycerides of lauric, myristic, palmitic, and stearic acids to be hydrolyzed with equal facility by pancreas lipase. Mazza and Valerie (36) however reported that stearic acid was hydrolyzed more readily by pancreatic lipase from 2,3-dimethyl-1-stearyl glyceride than from 1,3-dimethyl-2-stearyl glyceride although equilibrium was reached in both cases when 15% of the substrate was hydrolyzed.

In studies on the hydrolysis of triglycerides (monoacid) of saturated acids (C_8 to C_{13}) in the presence of pancreatic lipase, Holwerda (22) concluded that there was no difference in the rates of hydrolysis. On the other hand, Inoue and Sintani (23) reported the higher fatty acids of coconut oil are more readily hydrolyzed than the lower ones and that the saturated acids of cottonseed oil are hydrolyzed more readily than the unsaturated ones.

By way of conclusion, the results point to the need for employing fractionating devices capable of more complete resolution of the component glycerides in methods for determining glyceride distribution. Further work is also required to settle the question of relative rates of enzymatic esterification of different fatty acids with glycerol and rates of hydrolysis of the corresponding fatty acid esters. The present status of glyceride studies indicate that few if any fats have true random distribution. The general good accordance of experimental data with the restricted random principles merits further critical study. The principles of even distribution conform in a general or broad sense with the observed composition of many fats, particularly seed fats, but again more critical studies are required before any definite conclusions can be drawn as to their application to all types of fats.

REFERENCES

1. Ammon, R., and Jaarma, M., Chapter in "The Enzymes," vol. 1, p. 403, Academic Press, New York (1950).
2. Artom, C., and Zummo, C., Enzymologia, *3*, 231 (1937.).
3. Cama, J. S., Chakrabarty, M. M., Hilditch, T. P., and Meara, M. L., J. Sci. Food Agric., *4*, 321 (1953).

4. Collin, G., and Hilditch, T. P., J. Soc. Chem. Ind., *47*, 261T (1928).
5. Collin, G., and Hilditch, T. P., Biochem. J., *23*, 1273 (1929).
6. Dutton, H. J., Chapter in "Progress in the Chemistry of Fats and Other Lipids," vol. 2, Pergamon Press Ltd. (London), 1954.
7. Eckey, E. W., Ind. Eng. Chem., *40*, 1183 (1938).
8. Favarger, P., Helv. Physiol. et Pharmacol. Acta, *11*, C14 (1953).
9. Gunde, B. G., and Hilditch, T. P., J. Soc. Chem. Ind., *59*, 47T (1940).
10. Hilditch, T. P., "The Chemical Constitution of Natural Fats," 2nd ed., John Wiley and Sons Inc., New York, 1947.
11. Hilditch, T. P., J. Am. Oil Chemists' Soc., *26*, 41 (1949).
12. Hilditch, T. P., and Ichaporia, M. B., J. Soc. Chem. Ind., *57*, 44T (1938).
13. Hilditch, T. P., and Lea, C. H., J. Chem. Soc., 3106 (1927).
14. Hilditch, T. P., and Maddison, L., J. Soc. Chem. Ind., *59*, 162T (1940).
15. Hilditch, T. P., and Maddison, L., J. Soc. Chem. Ind., *59*, 67T (1940).
16. Hilditch, T. P., and Murti, K. S., J. Soc. Chem. Ind., *58*, 310T (1939).
17. Hilditch, T. P., and Murti, K. S., J. Soc. Chem. Ind., *60*, 16T (1941).
18. Hilditch, T. P., and Paul, S., Biochem. J., *32*, 1775 (1938).
19. Hilditch, T. P., and Pedelty, W. H., Biochem. J., *34*, 971 (1940).
20. Hilditch, T. P., and Stainsby, W. J., Biochem. J., *29*, 599 (1935).
21. Hilditch, T. P., and Stainsby, W. J., Biochem. J., *29*, 90 (1935).
22. Holwerda, K., Rec. trav. chim., *56*, 714 (1937).
23. Inoue, Y., and Sintani, G., Bull. Agr. Chem. Soc. Japan (in English), *17*, 68 (1941).
24. Jackson, F. L., and Longenecker, H. E., Oil and Soap, *21*, 73 (1944).
25. Kartha, A. R. S., Ph.D. thesis, 1949, University of Madras, published by the author, Ernakulam (1951).
26. Kartha, A. R. S., J. Am. Oil Chemists' Soc., *30*, 280 (1953).
27. Kartha, A. R. S., J. Am. Oil Chemists' Soc., *30*, 326 (1953).
28. Kartha, A. R. S., J. Am. Oil Chemists' Soc., *31*, 85 (1954).
29. Kartha, A. R. S., in press.
30. Kornberg, A., and Pricer, W. E. Jr., J. Biol. Chem., *204*, 345 (1953).
31. Longenecker, H. E., Chem. Reviews, *29*, 201 (1941).
32. Luddy, F. E., and Riemenschneider, R. W., Oil and Soap, *23*, 385 (1946).
33. Luddy, F. E., Fertsch, G. R., and Riemenschneider, R. W., J. Am. Oil Chemists' Soc., *31*, 266 (1954).
34. Luddy, F. E., Morris, S. G., Magidman, P., and Riemenschneider, R. W., J. Am. Oil Chemists' Soc., in press.
35. Mahler, H. R., and Wakil, S. J., J. Biol. Chem., *204*, 453 (1953).
36. Mazza, F. P., and Malaquzzi, Valerie, C., Arch. sci. biol. (Italy), *25*, 270 (1939).
37. Meara, M. L., J. Chem. Soc. *23* (1945).
38. Norris, F. A., and Mattil, K. F., Oil and Soap, *23*, 289 (1946).
39. Quimby, O. T., Wille, R. L., and Lutton, E. S., J. Am. Oil Chemists' Soc., *30*, 186 (1953).
40. Venkatarao, C., and Narasingarao, M., J. Indian Chem. Soc., *20*, 298 (1943).
41. Reiser, R., J. Am. Oil Chemists' Soc., in press.
42. Riemenschneider, R. W., Luddy, F. E., Swain, M. L., and Ault, W. C., Oil and Soap, *23*, 276 (1946).
43. Weber, H. H. R., and King, C. G., J. Biol. Chem., *108*, 131 (1935).